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"Respiratory Plasticity Following Spinal Injury: Role of Chloride-Dependent Inhibitory Neurotransmission"

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14. ABSTRACT The fundamental goal of our proposal is to test the hypothesis that spontaneous and induced plasticity in chloride-dependent synaptic inhibition of phrenic motor neurons contributes to functional recovery from chronic cervical spinal injuries. In the last year, we performed experiments to test the specific hypothesis that cervical spinal contusion injuries (CSC) and repetitive acute intermittent hypoxia (rAIH) shift the NKCC1/KCC2 balance in phrenic motor neurons, thereby degrading (CSC) and restoring (rAIH) chloride-dependent synaptic inhibition. Tissues were collected for immunocytochemistry and surface biotinylation/Western blot analysis and electrophysiology was performed on rats with sham surgery or CSC with and without AIH. We project that all tissues will be collected and all electrophysiological data will be obtained by December 12, 2014 for studies related to Specific Aims 1a, 1b, 2a, 2b. Our preliminary data suggest that membrane expression of KCC2 is reduced following CSC, which is normalized by rAIH treatment. Our data further suggest the surprising finding that rAIH effects on KCC2/NKCC1 balance may be reversed in the injured versus noninjured spinal cord. In the next year, we plan to complete electrophysiology and immunohistochemical/Western blot analyses, prepare a manuscript for publication and begin work on Specific Aims 1c, 1d, 2c.					
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## Annual progress report:

Award Number W81XWH-13-1-0410

"Respiratory Plasticity Following Spinal Injury: Role of Chloride-Dependent Inhibitory Neurotransmission"

Research Completed at University of Wisconsin, Madison

## Introduction

The fundamental goal of our proposal is to test the hypothesis that spontaneous and induced plasticity in chloride-dependent synaptic inhibition of phrenic motor neurons contributes to functional recovery from chronic cervical spinal injuries. In this project period, we will test the specific hypothesis that cervical spinal contusion injuries (CSC) and repetitive acute intermittent hypoxia (rAIH) shift the NKCC1/KCC2 balance in phrenic motor neurons, thereby degrading (CSC) and restoring (rAIH) chloride-dependent synaptic inhibition.

## SPECIFIC AIMS

**Aim 1:** Test the hypothesis that midline C4 cervical spinal contusions (CSC) degrade chloride-dependent inhibitory synaptic transmission in phrenic motor neurons by an atypical PKC-dependent mechanism, contributing to spontaneous functional recovery of breathing capacity.

- a. *Does CSC differentially alter membrane expression of the chloride co-transporters NKCC1 and KCC2 in the phrenic motor nucleus?*
- b. *Does CSC decrease GABAA receptor-induced inhibition of phrenic motor output?*
- c. *Does CSC-induced attenuation of chloride-dependent synaptic inhibition require PKC*
- d. *Is spinal PKC*

**Aim 2:** Test the hypothesis that repetitive acute intermittent hypoxia (rAIH) normalizes chloride-dependent inhibitory synaptic transmission in phrenic motor neurons by a TrkB-dependent mechanism.

- a. *Does rAIH normalize membrane NKCC1/KCC2 expression in the phrenic motor nucleus?*
- b. *Does rAIH normalize GABAA receptor-induced inhibition of phrenic motor output?*
- c. *Does rAIH-induced normalization of chloride-dependent synaptic inhibition require TrkB activity?*

## Overall project summary

Our strategy in this first year of this award was to collect rat tissues for immunocytochemistry and surface biotinylation/Western blot analyses and to begin our functional (electrophysiological) analyses of inhibitory neurotransmission. The laborious protein assessments are being made in the second year of the award.

In specific, we performed experiments in the following 4 groups of rats:

- Sham rats exposed to 4 weeks of normal oxygen conditions
- Sham rats exposed to 4 weeks of rAIH
- Rats with CSC exposed to 4 weeks of normal oxygen conditions
- Rats with CSC exposed to 4 weeks of rAIH

Overall in the first year, we successfully performed experiments on 57 rats. We are continuing to generate data, and project that all tissues will be collected and all electrophysiological data will be obtained by December 12, 2014 for studies related to Specific Aims 1a, 1b, 2a, 2b. Data for the immunocytochemical and Western blot analyses will be available after we complete our analyses for the specified proteins. Preliminary analyses of protein analyses are presented in figures 1 and 2. Data for the electrophysiological studies performed to date are summarized in figure 3.

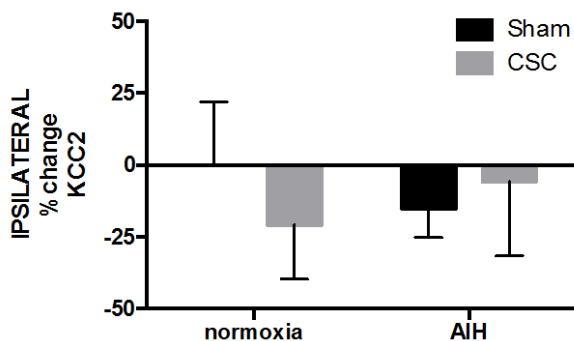
For immunocytochemistry, rats were anesthetized, perfused with paraformaldehyde, and the spinal cords were dissected. Tissues between cervical segments C4-C5 were sectioned with a microtome (40um sections) for analysis of KCC2 and NKCC1. We also sectioned the site of injury (C2) and stained the tissues with Cresyl violet to document the injury.

For surface biotinylation of membrane proteins, we collected ventral horn punches from spinal segments C4-C5. Tissues were suspended in cold buffer containing a cell-impermeable, cleavable biotinylation reagent (Sulfo-NHS-SS-Biotin). Biotinylated proteins were "pulled down" using Nutraavidin beads, and NKCC1/KCC2 levels were assessed in purified biotinylated (membrane) fractions and homogenates (both membrane and cytosolic components) with immunoblots. We also sectioned the site of injury (C2) and stained the tissues with Cresyl violet to document the injury.

For electrophysiology, rats were urethane-anesthetized, ventilated, paralyzed, and the phrenic nerve was recorded. We attempted to perform our electrophysiological assessments of inhibitory neurotransmission using nanoinjections of the GABA<sub>A</sub> agonist, muscimol, into the phrenic motor pool at C4. Our results were ambiguous, so we switched to intrathecal injections of muscimol over the C4 spinal segment, which gives us much more consistent results (summarized below). We initially chose a dose of 10 mM to deliver intrathecally; however, in the first round of rats, we realized that this initial dose was too high since phrenic motor output was abolished in all rats. Thus, we have since backed down to a dose of 7.5 mM. At this dose, we are able to detect a difference in inhibitory neurotransmission between groups (figure 3).

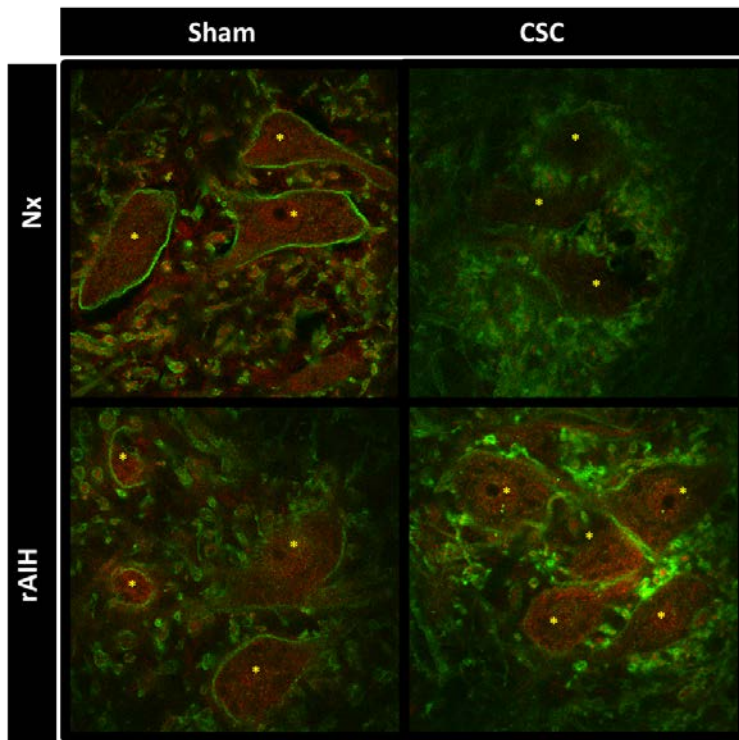
*Key accomplishments in the first year:*

- 1) Regulatory approval of all animal procedures was obtained (IACUC and ACURO).
- 2) New staff were trained to perform: 1) immunofluorescence for NKCC1 and KCC2 in fixed, spinal cord sections; 2) biotinylation and immunoblot analysis for NKCC1 and KCC2 in spinal cord tissues; 3) C2 lateralized contusion injuries; and 4) electrophysiology.
- 3) Latoya Allen attended the spinal cord injury training program at the Ohio State University to learn contusions in May 2014
- 4) We refined procedures for left lateral contusion injury and hemilaminectomy using the Infinite Horizons impactor at a force of 135 kD at the level of C3 rootlets.
- 5) A preliminary round of rats was collected to perform analyses on tissues from rats treated with: a) normoxia and sham surgery; b) normoxia and CSC; c) rAIH and sham surgery; and d) rAIH and CSC. Preliminary data are below (figures 1 and 2). Due to lessons learned, we refined techniques to standardize CSC and/or fresh tissue sampling. NKCC1 and KCC2 immunoblots from biotinylated cervical extracts (normalized to whole cell values) exhibited the following trends (Figure 1; each group n = 3): a) CSC decreased biotinylated (membrane) KCC2, without effect on NKCC1 (not shown); b) rAIH in sham rats appears to decrease membrane KCC2; but c) rAIH after CSC partially restored KCC2 to control levels without impact on NKCC1. These trends suggest that rAIH may have opposing effects on membrane KCC2 in the injured *versus* non-injured spinal cord.



**Figure 1:** Preliminary analysis of biotinylated KCC2 expression (membrane expression) in Western blots from tissues ipsilateral to injury. Biotinylated values are normalized to: a) whole cell lysate KCC2 expression, and then b) expressed as a percentage change from sham normoxia values. Samples were from ventral C3-C5 spinal segments (n=3 per group). These preliminary results suggest that: a) rAIH in sham rats decreases membrane KCC2 expression; b) CSC reduces KCC2 membrane expression; and c) rAIH after CSC at least partially restores membrane KCC2 expression to normal (sham normoxic) levels. Similar changes were not observed contralateral to injury. These results must be verified.

Photomicrographs of NKCC1 and KCC2 labeling in CtB labeled phrenic motor neurons (Figure 2; CtB not shown) confirm that NKCC1 is predominantly cytosolic in controls, whereas KCC2 is concentrated on motor neuron membranes. In these examples, CSC reduced KCC2 membrane levels, whereas rAIH partially restored it following injury. NKCC1 results were variable. Thus, these preliminary data using two distinct techniques support our fundamental hypothesis concerning the impact of CSC and AIH on KCC2 (but not NKCC1).



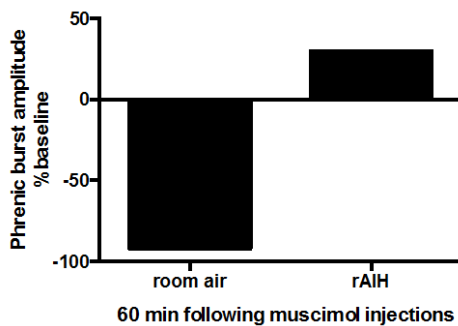
**Figure 2:** Immunofluorescence via confocal microscopy of NKCC1 (red), KCC2 (green) in C4 phrenic motor nucleus in normoxia and rAIH treated rats with and without CSC. All micrographs at 100X magnification from different rats. KCC2 expression is highly localized on the motor neuron membrane (indicated by small white asterisk; CtB was turned off in these micrographs to emphasize target proteins). It appears that CSC reduces KCC2 membrane expression and that rAIH at least partially restores it. NKCC1 results were highly variable (red), but suggest decreased cytosolic NKCC1 after CSC, and restoration with rAIH. Detailed quantitative analysis is underway.

- 6) A preliminary dose/response analysis was performed on 15 rats to determine the best delivery technique and dose of muscimol to maximize differences in inhibitory neurotransmission between groups. Based on these studies, we chose to intrathecally deliver of 7.5 mM muscimol to assess GABA<sub>A</sub> receptor-mediated neurotransmission.
- 7) New surgeries have been performed on 21 rats to complete the biotinylation assay related to Specific Aims 1a and 2a. Tissues have been collected from 10 of these rats; the remaining 11 rats are currently undergoing rAIH/room air treatment, and tissues will be collected within the next month. We plan to perform surgeries on rats needed to complete the study in the next two weeks, and tissues will be harvested by December 12, 2014. Tissues have been collected from the following groups to date:
  - sham + room air (n=2)
  - sham + rAIH (n=2)
  - CSC + room air (n=3)
  - CSC + rAIH (n=3)
 Western blot analysis on these tissues will be performed on October 30, 2014.
- 8) New surgeries have been performed on 23 rats to complete immunocytochemical experiments related to Specific Aims 1a and 2a. Tissues have been collected from 6 of these rats; the remaining 17 rats are currently undergoing rAIH/room air treatment, and tissues will be collected within the next month. We plan to perform surgeries on rats needed to complete this study in the next two weeks, and tissues will be harvested by December 12, 2014. Tissues have been collected from the following groups to date:
  - sham + room air (n=2)
  - sham + rAIH (n=2)
  - CSC + room air (n=0)
  - CSC + rAIH (n=2)
- 9) New surgeries have been performed on 13 rats to complete the electrophysiological experiments in Specific Aims 1b and 2b. Electrophysiological assessment of inhibitory neurotransmission has been performed on 4 of these rats; the remaining 9 rats are currently undergoing rAIH/room air treatment, and electrophysiological assessments will be performed within the next month. We plan to complete surgery on the remaining 19 rats needed to complete this study in the next two weeks, and electrophysiological assessments will be complete on December 12, 2014. Data from the following groups have been collected to date:

Sham + room air (7.5 mM dose): n=2

Sham + rAIH (7.5 mM dose): n=2

Data obtained from these four rats are shown in figure 3.



**Figure 3:** Phrenic burst amplitude 60 min following intrathecal muscimol (7.5 mM). In sham rats exposed to room air, phrenic burst amplitude decreases following muscimol injections, suggesting strong GABA<sub>A</sub> receptor-mediated inhibition. In sham rats exposed to rAIH, phrenic burst amplitude increases following intrathecal muscimol, suggesting that rAIH exposure degrades inhibitory neurotransmission. These results must be verified.

#### Problems:

We experienced a few issues in the past year that put us slightly behind schedule.

- Needed to train new personnel. Issue now resolved, all personnel are fully trained.
- Some rat death due to too severe of an injury using 200 kDa. Issue has been resolved; we are now using 135 kDa with a 5/6 survival rate.
- Some rat death following contusions, potentially due to contaminated central gas supply. Issue has been resolved.
- Several instances of impactor malfunction due to a short circuit in the computer board. Issue has been resolved; computer board has been replaced.

#### Key Research Accomplishments related to Statement of Work

*Task 1:* Obtain Animal and Human Use Approvals--Milestone accomplished

*Task 2: Specific Aims 1a and 2a:* Quantify changes in membrane NKCC1 and KCC2 expression in the phrenic motor nucleus following cervical contusion injuries in rats exposed to rAIH or room air.

*Subtask 1a.* Perform cervical contusion injuries and sham surgeries—94% complete.

*Subtask 1b.* Expose rats to rAIH beginning 1 week post-surgery—underway; projected completion date December 12, 2014.

*Subtask 1c.* Perfuse a subset of rats from subtask 1b 5 wks post-surgery and quantify changes in NKCC1/KCC2 using immunofluorescence—underway; projected completion date for tissue collection December 12, 2014. Preliminary data are summarized in figure 2 and bullet point #5 above. Laborious analyses and quantification will extend well into second year.

*Subtask 1d.* Harvest spinal cords from a subset of rats from subtask 1b 5 wks post-surgery and quantify changes in NKCC1/KCC2 using surface biotinylation and immunoblots—underway; projected completion date for tissue collection December 12, 2014; projected completion date for analysis of tissues January 1, 2014. Preliminary data are summarized in figure 1 and bullet point #5 above.

*Specific Aims 1b and 2b, Task 2:* Quantify GABA<sub>A</sub> receptor-induced inhibition of phrenic motor output with pressure microinjections of muscimol following cervical contusion injuries in rats exposed to rAIH or room air.

*Subtask 2a.* Perform cervical contusion injuries and sham surgeries—41% complete; remaining surgeries will be performed by November 7, 2014.

*Subtask 2b.* Expose rats to rAIH or room air beginning 1 week post-surgery—underway; projected completion date December 12, 2014.

*Subtask 2c.* Quantify phrenic responses to pressure microinjections of muscimol into the C4 phrenic motor pool using electrophysiology 5 wks post-surgery (all rats in task 2) —underway; projected completion date December 12, 2014. Preliminary data are summarized in figure 3 and bullet point #8 above.

*Subtask 2d:* Histological verification of extent of injury for all rats in task 2—underway.

## **Conclusions**

We have made good progress in accordance with our experimental plan, although we experienced an unexpected delay due to equipment malfunctions and a (possibly) contaminated gas supply. We project that all tissues will be collected and all electrophysiological studies related to Specific Aims 1a, 1b, 2a, 2b will be completed by December 12, 2014. Our major goals in the coming year remain to complete the laborious analyses of protein expression related to these aims, and begin studies related to Specific Aims 1c, 1d, 2c.

Although full results are not yet available, preliminary data are promising. Collectively, our preliminary results suggest that, consistent with our hypothesis, membrane expression of KCC2 is reduced by CSC, and is normalized by rAIH. This rAIH effect may minimize spasticity, while, at the same time, strengthen motor output. Additional preliminary data suggest the surprising idea that rAIH effects on KCC2/NKCC1 balance are opposite in injured versus noninjured spinal cords. These results may be highly significant with respect to future therapeutic treatments following spinal injury since literature reports suggest that rAIH is a novel, easy to apply and safe intervention that induces maximal functional benefits in chronic (versus acute) SCI. New treatments are crucial since currently available therapeutic interventions for spinal injury are severely limited in their efficacy; because of the variable and frustratingly limited functional recovery in breathing capacity, the major cause of death in patients with SCI continues to be respiratory failure. Strategies that effectively restore meaningful respiratory function go beyond increasing an SCI patient's quality of life--it is literally necessary save their life. Our research directly targets the goal of improving breathing capacity and/or coordination in patients with cervical SCI.

**Publications, abstracts and presentations:** None, pending completion of our studies.

**Inventions, patents and licenses:** None

**Reportable Outcomes**        None, pending completion of our studies.

**Other achievements:** None

**References**    None

**Appendices**    None